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<p>(21) International Application Number: PCT/US96/02662 (22) International Filing Date: 23 February 1996 (23.02.96) (30) Priority Data: 393,450 23 February 1995 (23.02.95) US (71) Applicant: UNIVERSITY OF UTAH RESEARCH FOUNDATION [US/US]; 210 Park Building, Salt Lake City, UT 84112 (US). (72) Inventors: REICHERT, W., Monty; 1500 Edgevale Road, Durham, NC 27701 (US). HERRON, James, N.; 2965 S. Plateau Drive, Salt Lake City, UT 84109 (US). WANG, Hsu-Kun; 73 Elizabeth Street #2, Salt Lake City, UT 84102 (US). CHRISTENSEN, Douglas, A.; 8520 Top of the World Circle, Salt Lake City, UT 84121 (US). (74) Agents: BOND, Laurence, B. et al.; Trask, Britt & Rossa, P.O. Box 2550, Salt Lake City, UT 84110 (US).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
<p>(54) Title: COMPOSITE WAVEGUIDE FOR SOLID PHASE BINDING ASSAYS</p> <p>(57) Abstract</p> <p>A step-gradient composite waveguide (100) for evanescent sensing in fluorescent binding assays comprises a thick substrate layer (102) having one or more thin film waveguide channels (104) deposited thereon.</p> <div data-bbox="939 1527 1889 1899"></div>		

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COMPOSITE WAVEGUIDE FOR SOLID PHASE BINDING ASSAYS

BACKGROUND OF THE INVENTION

Field of the Invention: The invention relates to apparatus for solid-state
5 biochemical binding assays, and especially to optical structures utilizing evanescent
sensing principles for use in such apparatus and assays.

State of the Art: Immunoassays exploiting the properties of an optical
technique known as total internal reflection (abbreviated TIR) are proving to be a
valuable tool for detection of analytes at concentrations of 10^{-10} to 10^{-13} molar or
10 below, without a wash step. When a light beam traveling in a waveguide is totally
internally reflected at the interface between the waveguide and an adjacent medium
having a lower refractive index, a portion of the electromagnetic field of the TIR
light penetrates shallowly into the adjacent medium. This phenomenon is termed an
"evanescent penetration" or "evanescent light". The intensity of evanescent light
15 drops off exponentially with distance from the waveguide surface.

Binding assays in general are based on the strong affinity of a selected
"capture" molecule to specifically bind a desired analyte. The capture
molecule/analyte pair can be an antibody/antigen pair or its converse, a
receptor/ligand pair or its converse, etc, as known in the art. In a fluorescent
20 binding assay, the binding of the analyte to the antibody is monitored by a tracer
molecule which emits fluorescent light in response to excitation by an input light
beam.

One of several possible schemes for exploiting the properties of evanescent
light fields for fluorescence measurements is as follows. If an antibody is
25 immobilized on an optical structure in which a light beam is being propagated by
TIR, the resulting evanescent light can be used to selectively excite tracer molecules
that are bound (whether directly or indirectly) to the immobilized antibody. Tracer
molecules free in solution beyond the evanescent penetration depth are not excited,
and therefore do not emit fluorescence. For silica-based optical materials or optical
30 plastics such as polystyrene, with the adjacent medium being an aqueous solution,
the evanescent penetration depth is generally about 1000 to 2000 Å (angstroms).
The amount of fluorescence is thus a measure of the amount of tracer bound to the
immobilized capture molecules. The amount of bound tracer in turn depends on the

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amount of analyte present, in a manner determined by the specifics of the immunoassay procedure.

U.S. Patents Nos. RE 33,064 to Carter, 5,081,012 to Flanagan et al, 4,880,752 to Keck, 5,166,515 to Attridge, and 5,156,976 to Slovacek and Love, and EP publications Nos. O 517 516 and O 519 623, both by Slovacek et al, all disclose apparatus for immunoassays utilizing evanescent sensing principles.

Desirably, an immunosensor should be capable of accurately and repeatably detecting analyte molecules at concentrations of 10^{-13} M (molar) to 10^{-15} M and preferably below. At present, such sensitivity is not believed to be available in a commercially practical and affordable immunosensor. Also desirably, an immunosensor should provide multiple "channels", that is, the capacity for measuring multiple analytes and multiple measurements of the same analyte, on the same waveguide substrate. Such an immunosensor would allow both self-calibration with known standards, and screening for a panel of different analytes selected for a particular differential diagnostic procedure.

One approach to improving the sensitivity (lowering the detection limits) of fluorescent immunosensors, proposed by Ives et al. (Ives, J.T.; Reichert, W.M.; Lin, J.N.; Hlady, V.; Reinecke, D.; Suci, P.A.; Van Wagenen, R.A.; Newby, K.; Herron, J.; Dryden, P. and Andrade, J.D. "Total Internal Reflection Fluorescence Surface Sensors" in A.N. Chester, S. Martellucci and A.M. Verga Scheggi Eds. Optical Fiber Sensors, NATO ASI Series E, Vol 132, 391-397, 1987), is to use waveguides which are very thin, perhaps about $1\mu\text{m}$ in thickness. Such thin waveguides may provide higher evanescent intensity and a reflection density of 500-1000 reflections/cm or more. However, the potential lowering of the detection limit by use of thin-film waveguides is achievable only if the waveguide material is nonfluorescent and low-loss. Most present evanescent immunosensing technology ("thick" waveguides) utilizes silica glass (SiO_2), which is intrinsically nonfluorescent. Only the purest grades of silica, for example UV grade quartz which is rather expensive, lack the additives and impurities that fluoresce (Dierker, et al., 1987).

Further, one cannot simply fabricate silica-on-silica waveguides by depositing SiO_2 onto a quartz substrate because there would be no refractive index difference. Instead one must either (1) fabricate a glass waveguide of higher

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refractive index than the underlying silica substrate, or (2) deposit a silica waveguide onto a transparent substrate of a lower refractive index. Therefore, other materials must be employed.

Thin film waveguides have been described by Sloper et al. ("A planar indium phosphate monomode waveguide evanescent field immunosensor, Sensors and Actuators B1:589-591, 1990) and Zhou et al. ("An evanescent fluorescence biosensor using ion-exchanged buried waveguides and the enhancement of peak fluorescence", Biosensors and Bioelectronics 6:595-607, 1991. However, neither of these devices was capable of achieving detection of analyte concentrations significantly below 10^{-10} molar. The waveguide structure of Sloper was of the gradient-index type, formed by diffusion of a dopant into the silica base, which results in a drop-off of dopant concentration with distance from the interface. The waveguide of Zhou had only a single "channel" (measurement region).

Therefore, a need exists for an optical structure useful in an evanescent sensing immunoassay, which provides increased levels of propagated TIR light and increased evanescent field intensity, as well as multiple measurement regions. Such an optical structure should desirably be capable of detection of analyte concentrations of 10^{-13} M and preferably below 10^{-15} M. A need also remains for an immunosensor including such an optical structure, which is sufficiently inexpensive and practical to be produced as a commercial device, and which provides accurate and repeatable results in the hands of non-skilled persons. Still further, a need also remains for a biosensor capable of detecting ions, as opposed to hormones or other biological molecules.

SUMMARY

The invention comprises a step gradient waveguide, also described as a composite waveguide, useful for performing evanescent sensing assays. The waveguide includes a thick substrate formed of a first optical material of refractive index n_1 and having a first surface, and a thin film formed of a second optical material having a refractive index n_2 which is greater than n_1 , the thin film being disposed adjacent and in operative contact with the substrate. The optical substrate has a thickness which may be from about $0.3 \mu\text{m}$ up to 10 mm or more, depending on the material used, while the thin film has a thickness which is generally between

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about 0.3 μm and about 5 μm . Highly preferably, the waveguide thickness is selected to provide for internal propagation in from one to four modes only.

The invention further encompasses a kit comprising the composite waveguide, and at least one specific binding molecule immobilized to said thin film
5 and constructed to bind with specificity an analyte. The kit may be further constructed for use in either a competition assay or a sandwich type assay. The tracer molecule is further constructed to be excited by evanescent light penetrating from the thin film into an adjacent aqueous environment, and to respond thereto by emitting a photodetectable tracer signal.

10 In a preferred embodiment, the composite waveguide comprises the substrate with a plurality of thin strips of the thin film disposed in parallel array thereon, and the kit further includes a second solution containing a known concentration of analyte in a buffer.

In preferred embodiments of the composite waveguide, coupling means are
15 integrally adapted and in operative contact with the thin film for coupling input light thereinto. One embodiment of coupling means is a grating etched into the substrate on the surface adjacent the thin film. Alternatively, instead of a physical grating a grating-type coupler may be composed of an array of segments of a different refractive index n_s disposed in the substrate in a regular spacing analogous to that of
20 the ridges in a grating. In another alternate embodiment, a relatively thick waveguide coupler is disposed on the planar surface of the thin film waveguide opposite the substrate, near one end of the composite waveguide. The waveguide coupler is dimensioned and constructed of appropriate optical material so as to evanescently couple light propagated by TIR in a thick input waveguide across a
25 thin spacing layer into the thin film waveguide.

In a highly preferred embodiment, the composite waveguide is constructed by vapor deposition of the thin film on the substrate, masking of the thin strips with a resist compound, and etching the thin film to expose the substrate in the unmasked areas. The resist compound is then removed to allow immobilization of the binding
30 molecules to the thin strips.

The invention further embraces apparatus for performing specific binding assays, the apparatus comprising a composite waveguide together with an optical unit having a light source positioned to direct light into the waveguide for

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propagation by total internal reflectance therein, and detection means oriented to detect light from a region proximal to the optical structure.

The IOW of the invention is capable of detecting analyte concentrations in the femtomolar (10^{-15} M) range. Such sensitivity is well beyond that achieved by other thin-film evanescent sensors, and also beyond the sensitivity expected solely on the basis of the increased reflection density intensity in the thin-film waveguide.

BRIEF DESCRIPTION OF THE DRAWINGS

In the drawings, which depict the best mode presently known for carrying out the invention:

FIG. 1A is a side cross-sectional view of the composite waveguide of the invention;

FIG. 1B is a top view of a composite waveguide module having a cross-sectional composition according to FIG. 1A;

FIG. 2A is a side view of a composite waveguide having an integral etched grating coupler;

FIG. 2B is a schematic illustration of the embodiment of FIG. 2A showing diffraction of normal incident light beam into a guided mode of the thin film waveguide;

FIG. 3A is a side cross-sectional view of an alternate embodiment of a composite waveguide, having an integral waveguide coupler;

FIG. 3B is a perspective view of the embodiment of FIG. 3A;

FIG. 3C is a schematic illustration of the overlap of the evanescent electromagnetic fields of the input waveguide and the thin film waveguide;

FIG. 4A is a schematic diagram of a top view with partial cutaway of a flow cell incorporating a multichannel composite waveguide;

FIG. 4B is a side cross-section taken along line B-B of FIG. 4A;

FIG. 5 is a schematic diagram of optical apparatus including the flow cell of FIGS. 4A and 4B, for performing binding assays therewith;

FIGS. 6A and 6B are charts of fluorescence measurements made with the apparatus of FIG. 4; and

FIG. 7 is a top view of an alternate embodiment of a composite waveguide, having etched sample wells.

DETAILED DESCRIPTION OF THE ILLUSTRATED EMBODIMENTS

A composite waveguide indicated at 100 has a substrate 102 formed of a first optical material of refractive index n_1 (FIG. 1A), and a thin-film waveguide layer 103 of a second optical material of refractive index n_2 , with n_2 being greater than n_1 . The waveguide layer 103 has a plurality of binding molecules 106, each constructed to specifically bind an analyte, immobilized on the upper surface 103A. The substrate 102 has a thickness D1 which may be from about 0.3 μm up to 10 millimeters (mm) or more, while the thin-waveguide 103 has a thickness D2 of between about 0.3 microns (μm) and 5 μm . If the substrate 102 is relatively thick, perhaps 0.5 mm or more, as shown in FIG. 1A, it may also serve as a mechanical support in addition to having the optical properties necessary for supporting effective waveguiding of light in the thin film waveguide 103. However, in some embodiments it is preferred, generally for easier manufacture, that the substrate 102 be comparatively thin. In such cases an additional support layer (not shown) may be added below the optical substrate 102 (not shown).

In a presently preferred embodiment, the substrate 102 is silicon dioxide, either in the form of a SiO_2 thin film laid down by vapor deposition or other techniques as known in the art, or in the form of quartz (here defined as including both natural quartz, and fused silica or other manmade quartz), with a waveguide layer formed of silicon oxynitride ($\text{Si}_2\text{O}_x\text{N}_y$, or, generally, "SiON"). The term "deposited SiO_2 " is used hereinafter to distinguish the deposited SiO_2 from quartz/fused silica, etc. Silicon dioxide has a refractive index of $n = 1.47$, and, if sufficiently pure, exhibits very low fluorescence. Silicon oxynitride is mechanically durable, transparent in the visible wavelength range, substantially non-fluorescent, and has a refractive index generally above 1.5 and reaching as high as 2.0 depending on the stoichiometric ratios of O to N. In a further presently preferred embodiment, the SiON is $\text{Si}_2\text{O}_3\text{N}$.

In another embodiment, the substrate 102 is formed of SiON having a refractive index n_1 and the waveguide film 103 is formed of SiON having a refractive index $n_2 > n_1$. The refractive index of the SiON compositions is controlled by the proportion of nitrogen. That is, the SiON of the substrate will have a lower proportion of nitrogen than the SiON of the waveguide film.

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In still another embodiment, the substrate 102 is formed of MgF_2 (magnesium fluoride), refractive index $n = 1.38$, and the waveguide layer 103 is a pure silicon dioxide thin film. Because the MgF_2 substrate is formed by vapor deposition techniques or the like which are tedious and comparatively expensive, in this embodiment the substrate 102 need be only sufficiently thick to ensure efficient propagation of at least one guided mode. At present, the SiON waveguide layer 103 is preferred, as it provides better adherence to the substrate in aqueous solutions than the MgF_2 waveguide. However, the MgF_2 waveguide/ SiO_2 composite waveguide may be entirely suitable for use with non-aqueous solvents.

10 In a presently preferred embodiment of a structure including a thin film SiON waveguide, the substrate 102 is a layer of SiO_2 deposited on a silicon wafer support. This is preferred because the surface of the substrate 102 is much more uniform and smooth, which in turn produces a much smoother planar surfaces in the deposited SiON film. The increased smoothness of the waveguide surfaces reduces the propagation losses by at least two-fold in comparison with a thin film waveguide deposited on a quartz or fused silica substrate.

In FIG. 1B, the waveguide layer is shown as a plurality of strips 104 which do not extend the full length of the substrate 102. This is an optional configuration of the waveguide layer designed to provide multiple waveguide "channels" for different sample solutions and/or for having different immobilized capture molecules. Further optionally, the composite waveguide 120 may be configured as a plurality of rectangular wells 108A, 108B, 108C, each containing three strips 104. The strips 104 are separated from one another by a distance D3 of at least about 5 mm. The wells 108A,B,C are separated by walls 110 extending upward from the substrate 102. The device of FIG. 1B may be formed by vapor deposition of the SiON over the entire surface of the substrate 102, masking the regions corresponding to the thin strips 104 with a resist compound, etching the exposed SiON to remove it from the substrate 102, and removing the resist compound. In FIG. 1B, shaded regions 112 represent areas of the waveguide which were not exposed to etchant. The binding molecules 106 are then immobilized to the thin strips 104. This can be accomplished by means known in the art of binding assays. Alternatively, the wells 108A, 108B, 108C may be configured for flow-cell type

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operation, as separate flow-through compartments for background, calibration (known analyte concentration), and test sample (unknown) solutions.

High quality composite waveguides made of the material combinations described above can be formed using plasma enhanced chemical vapor deposition (PECVD) to deposit a thin film over an entire surface of the substrate, and then lithographically etching away the thin film except in the desired strips. In the case of SiON, the PECVD is performed with a mixture of nitrogen, nitrous oxide, ammonia and silane gases, and the stoichiometry of $\text{Si}_2\text{O}_x\text{N}_y$ is varied by changing the respective partial pressures of the above components.

For the lithographic steps, it is presently preferred to use a reactive-ion etch (RIE) process with commercially available photoresist compounds. The areas of the film which are to become the strips or channels are coated with the resist, and the surrounding areas are etched down to the substrate. In the present embodiment, the DUV negative-tone photoresist XP89131 available from Shipley Co. is preferred, and the etchant is a plasma of O_2 and CHCl_3 gases. Positive tone resists such as XP2198 (Shipley Co.), APEX-E from IBM, and CAMP6 from OCG are also suitable. (All of these compositions are proprietary). All of these resist compounds are of the chemical amplification type, comprised substantially of phenolic polymers. While older photo-resists such as PMMA (polymethylmethacrylate) could be used, the chemically amplified DUV resists are considered to produce superior results for the present purposes.

Almost any other etching technique could be readily applied for etching the channels into the waveguide film, including plasma etch, ion milling, or a wet etch. However, where it is desired to use a substrate-etched grating to increase the coupling efficiency (see FIG. 2A and related description), the RIE process is preferred. This is because the etching process is critical to production of a highly anisotropic grating. RIE etching is believed to provide the best results for the grating, and wet etching would be unsuitable. For waveguides having an etched grating coupler, then, it is convenient to use the RIE process to etch the channels as well.

It is also within contemplation that the thin-film waveguide channels could be produced by a wet lift-off process. In this process, the substrate of the waveguide would be masked to leave the channel regions bare, and the SiON thin film would

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then be deposited over the whole surface including the masking agent. After deposition of the film, the entire surface would be immersed in a solvent selected to "lift-off" or remove the mask, together with the waveguide film deposited on the mask, while leaving the film deposited on the bare quartz.

- 5 All steps of the waveguide fabrication should be performed in an extremely clean environment, preferably a clean-room meeting the standards of at least class 10.

Example 1. SiON composite waveguide fabrication.

- 10 Waveguides comprising a 1 μm thick film of $\text{Si}_2\text{O}_3\text{N}$ on SiO_2 have been produced as follows. A heated sample holder containing a 10.16 centimeter (4 inch) quartz wafer was placed in a plasma-enhanced chemical vapor deposition (PECVD) reactor. Process gasses flowed from the perimeter of the PECVD vessel, over the sample, and were then pumped out of the vessel through a central port.
- 15 During deposition, the PECVD reactor was maintained at 300°C and 1.25 Torr, with 50W of power to a 13.56 MHz generator. The gas mixture consisted essentially of 27 standard cm^3 per minute (sccm) silane (SiH_4), 500 sccm nitrogen, 200 sccm ammonia, and 1300 sccm nitrous oxide. The respective inlet partial pressures were approximately 17 mTorr, 308 mTorr, 123 mTorr, and 802 mTorr.
- 20 Under these conditions, the deposition rate was about 590 Å/minute and a 1 μm film was produced in about 15 minutes. These silicon oxynitride films had an approximate elemental ratio of $\text{Si}:\text{O}:\text{N} = 2:3:1$, and a refractive index of about $n = 1.53$ to 1.54.

- Next, the $\text{Si}_2\text{O}_3\text{N}$ films were coated with photoresist, masked, and developed
- 25 to expose all but nine parallel 1 mm x 65 mm strips of the SiON film separated by 5 mm. The unmasked SiON was etched down to the quartz wafer. The photoresist was stripped, and the etched wafer was cleaved to produce three 23 mm x 69 mm rectangular pieces, where each piece contained three parallel 1 μm thick channel waveguides. Only the two outside channels were used in the assays described here.

- 30 There are several properties of composite waveguides which can be correlated to their relative suitability for evanescent-sensing biochemical binding assays. These include the ability to withstand the solvent used in the assay, which is generally but not always water; the amount of propagated light lost per unit

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distance traveled in the waveguide ("propagation loss", expressed as dB per cm; the intrinsic level of fluorescence of the waveguide upon irradiation with light of the excitation wavelength to be used, as measured within the bandwidth of the tracer fluorescence emission; reflections/cm at the waveguide-superstrate interface (N_r);

5 and adequate depth of evanescent penetration (d_p).

Example 2. Characterization of SiON waveguide.

Scanning electron microscopy was used to examine the shape and thickness of etched channel waveguides. A nominal channel should have optically smooth,

10 rectangular edges and a uniform thickness. For optical characterization of channel waveguides, the beam of the 632.8 nm line from a HeNe laser was coupled into the waveguide using a prism coupler. In a typical experiment, values were determined for waveguide thickness (t_{wg}), refractive index (n_{wg}), internal reflection angle (q_{wg}), reflections/cm at the waveguide-superstrate interface (N_r), depth of penetration (d_p),

15 and propagation loss (dB/cm). The data of Table 1 summarize the properties measured for a typical SiON waveguide constructed by the procedure outlined

Table I

Optical characterization of SiON waveguides

20	waveguide parameter	air superstrate	water superstrate
	t_{wg} (μm)	1.31 ± 0.11	N.A.
	n_{wg}	1.53 ± 0.0	N.A.
	propagation loss (dB/cm)	0.76 ± 0.09	N.D.
	q_{wg} (degrees)	82.67 ± 0.47	82.85 ± 0.42
25	d_p (nm)	44.10 ± 0.06	68.79 ± 0.29
	N_r (reflections/cm)	492.10 ± 71	479 ± 0.29

Each parameter was measured for three different channel waveguides with air as the superstrate. The mean and standard error of these

30 measurements is reported. In addition, the last three parameters (q_{wg} , d_p , N_r) were determined when water was substituted for air as the superstrate. N.D. - not determined.

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in Example 1. Also, these waveguides possess a high degree of physical definition and the desirable features of low propagation loss and minimal intrinsic waveguide fluorescence.

5 Example 3. Fabrication of MgF₂/SiO₂ composite waveguide.

The SiO₂/MgF₂ laminates were deposited in situ by electron beam evaporation, using a multipocket electron beam gun in a Balzers BAK760 high-vacuum coater. The deposition chamber was evacuated to 2 μ Torr, and the silica chips used as mechanical supports on which the laminates were deposited were
10 heated to 200°C. The chamber pressure rose to 3.8 μ Torr as a result of heating of the silica chips prior to deposition. The source materials, 99.9% pure MgF₂ and 99.999% pure SiO₂, were placed in molybdenum- and graphite-lined hearths, respectively, in the rotatable source carousel. Both source materials were brought to the deposition temperature with the shutter closed. First the MgF₂ was
15 evaporated with a slowly sweeping 10-kV (kilovolt) electron beam (\approx 12 mm² elliptical spot size) followed by similar evaporation of the SiO₂. The deposition rates of MgF₂ and SiO₂ were controlled at 20 Å/s and 10 Å/s (angstroms per second), respectively, by means of an oscillating crystal monitor. A 0.36 μ m film of MgF₂ was deposited in 3 minutes, and a 1.0 μ m film of SiO₂ was deposited in 16
20 minutes.

Following construction of the physical portion of the waveguide, a plurality of specific binding molecules, that is, molecules having the property of specifically binding a chosen analyte, are immobilized on the surface of the thin film waveguide channel(s). Such specific binding molecules may be antibodies, receptor molecules,
25 and the like, or fragments thereof that are operative to specifically bind the corresponding analyte. Converse pairs, e.g. such as an antigen for detecting certain antibodies with the analyte being the antibody, are also suitable. Other types of binding molecule/analyte pairs will be apparent to those of ordinary skill, as will means for immobilizing the binding molecule. Presently preferred means for
30 immobilizing the binding molecule are discussed subsequently herein.

A flow cell incorporating a two channel waveguide fabricated and characterized as in Examples 1 and 2 is shown in FIGS. 4A and 4B. A composite waveguide 100 is held between a bottom plate 402 and a top plate 404 spaced by a

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pair of black silicone rubber o-ring gaskets 406 (FIG. 4B). Each gasket is seated in a corresponding groove 406A milled in the top plate, and forms a longitudinal flow channel 408. The flow channels have respective inlet ports 410A, 412A and outlet ports 410B, 412B. Arrows 409 indicate the direction of flow of liquid through the channel 408. Each flow channel also has a hole 414 for placing a coupling prism 416. The interior of the bottom plate has a window 418 milled therein to support the waveguide and provide a clear view of the waveguide bottom through the silica chip support layer. The aluminum parts of the entire flow cell are desirably flat black, to absorb stray light. Coupling prisms (4 mm wide by 10 mm high truncated 45-45-90 LaSF prisms, $n_p = 1.83$, obtained from Karl Lambrecht Co. were fixed in place inside the windows 414 with GTE 118 RTV silicone rubber cement. A series of finger screws (not shown) were used to clamp the top and bottom plates 404, 402 to the substrate 102, and to maintain coupling pressure on the prisms 416. Polyethylene screw-in tubing connectors, TEFLON[®] microtubing, and disposable syringes served as the sample delivery system (not shown). The sample volume of each channel 408 was 300 μ L.

Prism coupling the waveguide with aqueous superstrate comprises placing the waveguide on the bottom plate of the flow cell, wetting the waveguide surface opposite the substrate with buffer solution to be used in measurements, and then placing the top plate with the attached prisms over the wetted waveguide. An advantage of the raised-channel waveguide structure is that effective prism coupling is easily accomplished by placing mild pressure on the coupling prisms.

Evanescent binding assays in two model systems were performed with the flow cell of FIGS. 4A, 4B, using a dual-channel assay format similar to that described by Herron et al. in "Fluorescent immunosensors using planar waveguides", SPIE Vol. 1885 (Advances in Fluorescence Sensing Technology), pp. 28-39, 1993, as well as in the copending U.S. Patent Applications Serial Nos. 08/064,608 and 08/263,522.

30 Example 4. Model assays using thin-film waveguide flow cell.

In the first or direct-binding model system, the binding of a biotinylated antibody labelled with a fluorescent tracer to avidin immobilized on the waveguide surface was measured. The particular antibody used was an anti-fluorescein

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antibody designated 9-40, produced by standard hybridoma methodology. The 9-40 antibody was labelled with the dye Cy-5 ($\lambda_{\text{abs,max}} = 650 \text{ nm}$, $\lambda_{\text{em,max}} = 667 \text{ nm}$, $\epsilon_{\text{max}} = 2 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$) according to the manufacturer's procedure (Biological Detection Systems). The labeling efficiency was approximately four dyes per protein molecule. An untreated hydrophilic SiON IOW surface was treated for 8 hr with a 0.2 mg/mL solution of avidin (obtained from Sigma) dissolved in phosphate buffered saline (PBS, pH 7.4), to physically adsorb the avidin to the waveguide surface.

A portion of the Cy-5 labeled 9-40 was biotinylated using N-hydroxy-succinimidobiotin (Sigma). Specifically, a 20-fold molar excess of this reagent was added to a 1 mg/mL solution of 9-40 in 0.1 M sodium carbonate/sodium bicarbonate buffer, pH 9 (CBB). This mixture was allowed to react for 2 hr at room temperature, and the product (biotinylated, CY-5-conjugated 9-40) was purified by gel permeation chromatography using a PD-10 column (Pharmacia) equilibrated in PBS.

The second type of assay was an indirect, sandwich type assay in which the binding of free fluorescein-conjugated BSA to immobilized anti-fluorescein antibody (antibody 9-40) was measured using an anti-BSA antibody conjugated with Cy-5 as a tracer. The IOW surfaces with immobilized 9-40 antibody was prepared as follows. Prior to immobilization, 9-40 was acid pre-treated in citrate buffer (pH 3) for 1 hr and reconstituted in PBS at a concentration of $2.3 \times 10^{-7} \text{ M}$ (0.03 mg/ml), following the procedure described by Lin et al. (1989). SiON IOWs were treated with 1% dichlorodimethylsilane (DDS, Sigma), rinsed three times in deionized water, and then immersed in a solution of the acid-pretreated antibody 9-40 for 3 hr at room temperature.

Murine monoclonal anti-bovine serum albumin antibody (anti-BSA; available from Sigma) was labeled with Cy-5 as described for antibody 9-40. Bovine serum albumin (Sigma) was labeled with fluorescein isothiocyanate (FITC, Sigma). A 20-fold molar excess of FITC was added to a 1 mg/ml solution of BSA in CBB. This mixture was allowed to react for 1 hr at room temperature, and the conjugate was purified using a PD-10 column equilibrated in PBS. Labeling efficiency was approximately 2 FITC groups per BSA molecule. It should be noted that since the assays were performed with excitation at 632.8 nm, a wavelength which well

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exceeds the absorption band of FITC ($\lambda_{\text{abs,max}} = 492 \text{ nm}$), the FITC here serves solely as a hapten for 9-40, and not as a fluorescent tag.

To perform the assays, IOWs coated as described in the preceding paragraphs were mounted in a flow cell like that of FIG. 4, and placed on a rotating
5 goniometer (not shown) in the collection path of a spectrograph 502 associated with a CCD camera 504 (FIG. 5). The goniometer is optional, but useful in an experimental set-up to ascertain the best coupling angle for coupling the laser beam into the waveguide. Once the best coupling angle has been determined and the apparatus standardized, a goniometer is not required to perform the assay. The
10 input light was the red line (6 mW at 632.8 nm) of a HeNe laser (made by Melles Griot), split into dual beams 506, and simultaneously prism coupled into the two channels 104 of the composite waveguide. In this manner, a reference channel and a sample channel were excited.

For detection, the 1 mm wide guided modes in each channel were oriented
15 perpendicular to the entrance slit of the spectrograph. Light emitted from a 1 mm long section of the two parallel streaks was imaged through the window 418 in the bottom plate 402 of the flow cell with a 50 mm f/5.6 camera lens onto the slit of spectrograph 502. The collected light emission was wavelength-dispersed using a single grating monochromator 502 (SPEX 1681c Minimate-2, f/3.9, 300
20 grooves/mm) centered at 700 nm, and directed onto a thermoelectrically-cooled CCD detector 504 (Photometrics Series 200), producing an image 508 containing reference region 510 and sample region 512. The reference and sample image regions 510, 512 were separately binned and vertically integrated and converted on a MACINTOSH IIfx computer to reference spectrum 520 and sample spectrum 522,
25 respectively, of intensity vs. wavelength.

Both assays followed the same general scheme: 1) a capture protein was immobilized to the surface of both the sample and reference channels; 2) aliquots of the hapten-carrier protein conjugate (with fluorescent tracer) were added to the sample channel; and 3) the same concentration of fluorescent tracer (without hapten)
30 was added simultaneously to the reference channel.

Each measurement began by collecting the baseline spectra of PBS buffer in both the sample and reference channels. Starting with the most dilute analyte solution, 1 mL of the sample and reference solutions described in Table 1 were

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injected into the flow cell. The solutions were incubated with the waveguide surface for 2 min for assay 1 and 5 min for assay 2, followed by collection of 10 sec spectrograph images of both channels. This process was repeated several times until the most concentrated solution was assayed. All CCD images were collected
5 without a wash step; i.e., with the fluorescent analyte filling the sample and reference channel volume. Reference and sample spectra derived from each CCD image of each measurement were integrated from 653 nm to 693 nm. Binding curves were constructed from these data by taking the ratio of the integrated intensity of the sample to that of the reference, and then plotting these ratios as a
10 function of bulk analyte concentration. Such radiometric measurements compensated for two effects: nonspecific binding of the tracer, and parasitic excitation of bulk fluorescence.

The results obtained with the two assay schemes are shown in FIGS. 6A-6B. The results indicate that analyte could be detected at concentrations as low as
15 3×10^{-15} M with a two-channel $\text{Si}_2\text{O}_3\text{N}$ thin film waveguide flow cell. These measurements are at least 3.3×10^4 times more sensitive than the thin film waveguide immunosensors reported by Sloper et al. or Zhou et al., *op cit.*, and at least 33 times more sensitive than the thick film sensor of Herron et al. 1993, *op cit.*

The prism coupling scheme described in Example 4 is efficient, simple, and
20 well suited for use in laboratory experiments. However, such a device is relatively expensive, and thus less than ideal for a disposable IOW module for use with a point-of-care optical unit. Further, the prism coupling scheme requires precise pressure on the prisms and precise alignment of the incident laser beam.

An alternate embodiment of a coupling scheme, which avoids these
25 problems, is to provide a diffraction grating etched either into the surface of the waveguide opposite the substrate, or into the substrate adjacent the waveguide. The presently preferred embodiment is that of a substrate-etched grating. As seen in FIG. 2A, the substrate 102 has a grating 200 etched into the surface 102A which is in contact with the thin film 103. The binding molecules 106 are immobilized
30 down-beam of the grating 200. Optionally, a second grating (not shown) may be etched into the substrate 102, down-beam of the immobilized binding molecules. Such a second grating is for purposes of optical characterization of the waveguide, and is not required for purposes of the fluorescence binding assay.

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The depicted substrate-etched grating is advantageous in that the light can be conveniently in-coupled from the back side of the device, and without interference from macromolecules bound to the waveguide surface. Also, it is convenient and economical from a manufacturing perspective to etch the grating into the substrate prior to deposition and etching of the channel waveguides.

The efficiency of light incoupling by the etched grating will vary according to the spacing, the profile or blaze, and the aspect ratio of the grooves. These parameters should be optimized according to the wavelength of the input (excitation) light, the index of refraction of the superstrate, and the waveguide thickness, by techniques conventional in the art. In the present case, a grating period D_4 of about $0.42\ \mu\text{m}$ was computed for coupling of a normally incident $632.8\ \text{nm}$ laser beam into the $m=0$ mode of the thin film SiON waveguide ($\Theta = 83^\circ$; see FIG. 2B).

Still another coupling scheme uses the principle of evanescent penetration to evanescently couple light from a waveguide coupler into the composite waveguide across a precise spacing layer (FIGS. 3A, 3B and 3C). The waveguide coupler is contactingly mounted on the upper surface of the composite waveguide, adjacent the exposed surface of the thin film. Like the prism coupler, the waveguide coupler does not extend into the region to which the specific binding molecules are immobilized.

As seen in FIG. 3A, a waveguide coupler 300 comprises an input waveguide 302 which consists of a comparatively thick (1 mm or greater) layer of material of refractive index n_3 , and a thin spacing layer 304 of refractive index n_4 , where n_4 is less than both n_3 and n_2 . The input waveguide has a receiving edge for receiving the input laser light, and a thickness D_5 which is sufficient to allow easy end coupling of the input beam into the receiving edge. The spacing layer has a thickness D_6 selected to maximize the evanescent coupling of waveguided light from the input waveguide into the thin film of the composite waveguide.

According to coupled mode theory, light propagating in one waveguide can be synchronously coupled into an adjacent waveguide if there is overlap between the evanescent regions of the two waveguides in the low index region separating them (FIG. 3C). The efficiency of coupling depends upon the spacing between the guides, the respective modal propagation constants, and the distance of the interaction. Since the coupling efficiency varies according to the cosine of the

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distance f interaction, in theory it will reach 100% at a certain length. Therefore, the synchronous evanescent coupling device should be quite efficient.

To achieve maximum coupling efficiency, the spacing layer thickness should be adjusted such that

$$5 \quad K_1 = (j + 1)\pi/2, (j = 0, 1, 2\dots)$$

where K is the coupling constant and depends on the refractive indices n_1, n_2, n_3, n_4 , the wavelength λ of the input light, and the respective thicknesses D_2, D_5 and D_6 of the thin film waveguide, the input waveguide, and the spacing layer. With the thin film waveguide, it should be taken into account that generally, only one or
 10 a few of the lowest modes (preferably from $j=0$ to about $j=10$, and no more than $j=20$) are being propagated in the waveguide. Preferred waveguide couplers are configured to increase the proportion of laser light which is propagated in these lower modes.

For a silicon oxynitride composite waveguide, the spacing layer can be made
 15 of SiO_2 . Desirably, the spacing layer 304 is deposited epitaxially, so that its thickness can be controlled very accurately at the time of deposition. The input waveguide 302 is attached on top of the spacing layer, after all epitaxial processing and etching steps have been finished in the fabrication of the composite waveguide. The input waveguide is made of a high refractive index material such as rutile,
 20 zirconia, or a high-index glass, and may be adhered to the spacing layer 302 by an index-matching cement of refractive index near n_3 . This assembly would be comparatively inexpensive, and is thus attractive for a disposable, one-time use module.

Preferably, as shown in FIG. 3A, the laser light is focussed into the
 25 receiving edge of the input waveguide at an angle Θ' which is a degree or two less than the critical angle for TIR at the interface between the input waveguide and the spacing layer. Such angled beam entry has been found to substantially increase the proportion of internally propagated light in the evanescent tail, and thus the evanescent intensity.

30 Still another embodiment is an IOW with integrally formed sample wells 702, 704, 706 (FIG. 7), each of which may contain multiple thin waveguide channels 104. In a preferred embodiment, walls 700 which enclose the wells are formed by layering a submicron cladding film of the substrate optical material on

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the composite waveguide, appropriately masking, and then etching the cladding back down to the SiON waveguide layer. These steps are performed prior to immobilization of the specific binding molecules. Sample wells thus constructed provide physically defined regions in which either different antibodies can be immobilized, or to which different solutions, e.g. background or control samples vs. test samples, can be added. The same technique could be used to form open-ended longitudinal flow channels instead of the box-shaped reservoirs illustrated. Also, instead of layering the reservoir wall material over the composite waveguide, the same technique could be used to form reservoir or flow channel walls on a separate top plate.

At present it is believed that two additional factors related to the chemistry of immobilization of specific binding molecules to the waveguide surface can affect the sensitivity achieved. These are 1) the relative amount of non-specific protein binding to the thin-film surface (including the walls of any sample wells); and 2) the analyte/tracer-binding capacity of the immobilized binding molecules.

In a highly preferred embodiment of the multi-channel IOW, the IOW surface is treated to minimize non-specific binding which otherwise can cause significant overlap of tracer signal between channels. The problem of such overlap worsens rapidly with increasing numbers of channels per IOW. Take as an example a six-channel waveguide divided into three adjacent-channel pairs in three corresponding separate wells, with anti-CK-MB immobilized to one member of each pair and anti-CK-MM immobilized to the other member. The IOW so constructed would permit simultaneous calibrated measurement of binding of 2 different analytes, CK-MB and CK-MM. If the level of non-specific binding is assumed to be about 5 % of the specific binding level, the channel with anti-CK-MB covalently attached will be contaminated to about 5 % with non-specifically adsorbed anti-CK-MM. Conversely, the channel with anti-CK-MM will be contaminated to about 5 % with non-specifically adsorbed anti-CK-MB. Thus, with such a 2-analyte IOW there could be about 10% crosstalk between the channels, already an undesirable contribution to background level. For a 4-analyte IOW (twelve channels), however, the background contributed by such crosstalk would increase to about 30%, which would seriously impair detection sensitivity. Obviously the problem worsens with increasing numbers of different analytes.

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For this reason, it is highly desirable that the channels be coated with a coating that reduces non-specific binding to 1 % or less of specific binding, prior to covalent immobilization of the specific binding molecule.

A method of enhancing the number of available analyte binding sites is to site-specifically immobilize the analyte binding molecules. For antigen-binding fragments such as $F(ab')_2$'s, this can be accomplished by providing a base coating having pendant maleimido groups. The $F(ab')_2$ fragments are then reduced to yield Fab' fragments which contain a free thiol group at their C-terminal end, and this thiol group readily reacts to covalently couple the $F(ab')_2$ fragment to the maleimido group. Desirably, the coating with pendant maleimido groups also provides the inhibition of non-specific binding discussed in the preceding paragraph; this may for example be accomplished by a base coating comprising chains of polymerized hydrophilic residues adhered via a silica-affinic moiety to the waveguide surface, as disclosed by the copending U.S. patent applications S.N. 08/064,608 and 08/263,522. Protocols for achieving both of these methods can be found in the noted copending applications, and in the literature in Herron et al., "Fluorescent immunosensors using planar waveguides", *SPIE* 1885:28-39, 1993. Preferably, at least 70% of the immobilized analyte binding molecules should be available for binding.

Introduction

Nucleic acid probes are gaining increasing acceptance in clinical diagnostics and may one day equal or surpass the importance of immunoassays. Because of their exquisite sensitivity, evanescent wave sensors have the potential of being able to detect DNA hybridization without prior amplification of the analyte. The strategy of this invention is to employ the nucleic acid analog of an immunological sandwich assay. Specifically, an oligonucleotide primer (capture oligonucleotide) complementary to the sequence of interest (analyte) is immobilized to the waveguide using either amine-reactive, thiol-reactive or the (strep)avidin-biotin coupling chemistry (see Herron, J. N., Caldwell, K. D., Christensen, D. A., Dyer, S., Hlady, V., Huang, P., Janatova, V., Wang, H.-K., and Wei, A.-P. "Fluorescent Immunosensors using Planar Waveguides," *SPIE* Vol. 1885 (Advances in Fluorescence Sensing Technology) 28-39 (1993).

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Next, a fluorescent label such as Cy-5 is coupled to one end of a second oligonucleotide primer (tracer) which is complementary to another sequence on the analyte. To perform the assay, the analyte is first heated if it is double-stranded (to separate the two strands) and then mixed with the tracer oligonucleotide; this mixture is delivered to the sensor where the analyte hybridizes with the capture nucleotide and brings the tracer oligonucleotide into the evanescent field, where it (the tracer) fluoresces.

For detection of nucleic acids, the major technical barrier is the elimination of the amplification step (e.g., polymerase chain reaction), which is required in present-day assays to increase the amount of nucleic acid up to a measurable level. In theory, evanescent wave sensors possess the intrinsic sensitivity necessary to measure non-amplified DNA. However, there are many technical barriers to achieving this goal including development of (1) immobilization strategies which give very low levels of non-specific binding (perhaps 10- to 100-fold lower than present day technology); (2) effective reagent dispensing strategies that can deliver femtogram quantities of nucleic acid to the sensor surface without losses in a reasonable amount of time (5-10 min); and (3) effective strategies for denaturing double-stranded analytes into the single-stranded form required for hybridization assays.

20

Materials and Methods

Waveguides

Planar waveguides are employed for conducting fluorescent nucleic acid hybridization assays. Nucleic acid probes are immobilized as described below to either fused silica waveguides (1.0 x 1.0 x 0.1 cm, CO grade, ESCO) or injection molded polystyrene waveguides (1.0 x 1.0 x 0.05 cm, HCP Diagnostics, Salt Lake City).

25

Nucleic Acid Probes

The transcriptional promoter site of the T3 RNA polymerase is employed as a model oligonucleotide sequence in these studies. The T3 promoter is a 20-mer consisting of the following sequence: 5' AATTAACCCTCACTAAAGGG 3'. This oligonucleotide is synthesized and purified by a molecular biology service facility at

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the University of Utah. When used in DNA hybridization assays, it is immobilized to waveguides (using procedures described below) and used as the "capture" oligonucleotide. It is also used as the soluble inhibitor in competition assays. A second oligonucleotide sequence, complementary in sequence to the T3 promoter, is synthesized at the same service facility, fluorescently-labeled and used as the soluble "tracer" oligonucleotide in DNA hybridization assays. To this end, the oligonucleotide is synthesized with a terminal amino group on the 3' end and labeled with Cy-5 (Molecular Detection Systems, Pittsburgh), a red-emitting fluorescent dye.

10

Immobilization of oligonucleotide probes to silica waveguides

Oligonucleotide probes are coupled to silica waveguides using the avidin/biotin coupling chemistry. The general strategy is to immobilize avidin to the waveguides via electrostatic interactions (silica is negatively charged and avidin positively charged at neutral pH) and then complex biotinylated nucleotides to the immobilized avidin. Biotinylated oligonucleotides are prepared by coupling biotinamidocaproate N-hydroxysuccinimide ester (BCHS, Sigma) to a modified oligonucleotide that contained a 5' amino group. The BCHS derivative is used in order to provide a six carbon spacer between the 5' end of the oligonucleotide and the biotin moiety. The spacer is thought to provide the oligonucleotide with more conformational flexibility in hybridizing with other nucleotides. Specifically, a 20-fold molar excess of BCHS is added to a 0.1 mg/mL solution of oligonucleotide in 0.1 M sodium carbonate/sodium bicarbonate buffer, pH 9 (CBB). This mixture is allowed to react for 2 hr at room temperature, and the product (biotinylated oligonucleotide) is purified by reverse phase FPLC chromatography (Pharmacia) using an acetonitrile/H₂O gradient. Avidin is immobilized to silica surfaces by physical adsorption. To this end, clean silica samples are immersed in an avidin solution [3×10^{-6} M in phosphate buffered saline (PBS), pH 7.4] for 3 hr. at room temperature and then washed several times in the same buffer to remove unabsorbed avidin. Avidin-coated surfaces are then immersed in a 1.5×10^{-7} M solution of the biotinylated oligonucleotide in PBS for 1 hour at room temperature. Unbound nucleotide is removed by washing in PBS. In some cases, biotinylated poly(ethylene glycol) (biotin-PEG) is coupled to surfaces following the

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immobilization of biotinylated oligonucleotides. Biotin-PEG is prepared by reaction of N-hydroxysuccinimidobiotin (NHSB) with $\text{NH}_2\text{-PEG-OCH}_3$ (5000 MW, Sigma) for 2 hours in CBB, and purified by dialysis using Spectra/Por dialysis membranes (1000 MW cutoff). Silica surfaces (coated with avidin and oligonucleotides) are
5 immersed in a solution of biotin-PEG (5×10^{-8} M or 1×10^{-7} M) for 1 hour at room temperature. Unbound biotin-PEG is removed by washing in PBS.

Immobilization of oligonucleotide probes to injection molded polystyrene waveguides

10 Similar procedures are employed for immobilizing oligonucleotides to polystyrene waveguides, except that streptavidin is used instead of avidin. Whereas avidin interacts strong with silica substrates due to electrostatic interactions, our experience has shown that it does not adsorb nearly as well to polystyrene surfaces (which are uncharged and hydrophobic in nature). In contrast, streptavidin is the
15 protein of choice for adsorption to polystyrene surfaces. Although functionally equivalent to avidin (i.e., it also contains four biotin binding sites), streptavidin possesses little charge at neutral pH and retains its biotin binding activity upon adsorption.

20 Total internal reflection fluorescence measurements

 Total internal reflection fluorescence (TIRF) spectroscopy is an optical technique that is especially well-suited for measuring the concentration of fluorescent molecules attached to a solid surface such as a waveguide. Because water has a lower index of refraction than either silica or polystyrene, a beam of
25 light traveling through a waveguide and striking its edge will either be refracted into the aqueous phase or reflected totally back into the waveguide, depending on the angle of incidence. In the latter case, the incident and reflected beams interfere to produce a standing wave in the waveguide. This wave has a finite electric field amplitude at the edge of the waveguide, but decays exponentially over a distance of
30 1000 to 2000 angstroms as one moves into the aqueous phase. This decaying electric field is referred to as the evanescent field.

 Because the evanescent field is confined relatively close to the waveguide's surface, it can be used to detect nucleic acid hybridization reactions which occur at

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the surface. Waveguides are coated with oligonucleotides as described above and then mounted in the flowcell described in co-pending patent application serial no. 08/064,608. The fluorescence of the Cy-5 labeled tracer oligonucleotide is excited with either the 632.8 nm line of a He-Ne laser (Melles Griot) or the 633 nm line of a semiconductor laser (Power Technology, Inc.). The red line is formed into a sheet beam (with a cross-sectional area of ca. 0.2 cm x 3 cm) and coupled simultaneously into the two channels (sample and reference) of the waveguide using a cylindrical coupling lens. The fluorescence emission of Cy-5 is collected using a 50 mm f/5.6 camera lens (Nikon) attached to a charge-coupled device (CCD) (either Photometrics Ltd Series 200 or Santa Barbara Instrument Group ST6). Fluorescence emission is discriminated from Rayleigh light scattering using either a single grating monochromator (SPEX 1681c Minimate-2, f/3.9, 300 grooves/mm) or a 670 nm bandpass interference filter (Omega).

15 Assay Scheme

The use of planar waveguides and TIRF in detection of DNA hybridization is evaluated in two different assays. The first of these (Assay 1) is a direct binding assay in which one strand of a DNA duplex is immobilized to the waveguide (referred to as the "immobilized strand") and the other strand (referred to as the "soluble strand") is labeled with a fluorescent dye. Formation of double-stranded DNA molecules on the waveguide is monitored as a function of the concentration of fluorescently-labeled soluble strand. The second assay (Assay 2) is a competition assay based on the same reagents as described in Assay 1. In this case, however, both labeled and unlabeled species of the soluble strand are present. Specifically, different concentrations of the unlabeled soluble strand are mixed with a fixed concentration of the labeled soluble strand and each mixture is allowed to react with the immobilized strand on a waveguide sensor. The formation of double-stranded DNA molecules is monitored using TIRF. Table I lists the reagents used in the two assays.

30 Assays are performed using the two-channel flowcell described in co-pending patent application serial no. 08/064,608. A "sample" solution containing the reagents described above is injected into one channel and a "reference" solution is injected into the other (see Table I for a description of sample and reference

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solutions employed in each assay). The reference solution is used to measure the degree of non-specific binding between the fluorescently-labeled oligonucleotide and the waveguide and also to correct for fluctuations in laser intensity that occurred during the course of an experiment. Each measurement begins by collecting the

5 baseline fluorescence of PBS buffer in both the sample and reference channels. Starting with the most dilute analyte solution, 1 mL of the sample and reference solutions described in Table 1 are injected into the flowcell. The solutions are allowed to incubate with the waveguide surface for 5 minutes, followed by collection of 10 sec fluorescence images of both channels. This process is repeated

10 several times until the most concentrated solution is assayed. All CCD images are collected without a wash step; i.e., with the fluorescent analyte filling the sample and reference channel volume. Binding curves are constructed from these data by taking the ratio of the intensity of the sample to that of the reference, and then plotting these ratios as a function of bulk analyte concentration. Such radiometric

15 measurements compensate for three effects - nonspecific binding of the tracer, excitation of bulk fluorescence by light scattered off the surface of the waveguide and fluctuations in laser intensity.

It will be apparent that details of the composite waveguide can be varied considerably without departing from the concept and scope of the invention. The

20 claims alone define the scope of the invention as conceived and as described herein.

THE CLAIMS**What is claimed is:**

1. A kit for performing specific binding assays with an optical unit including a light source positioned to direct light into an optical substrate and
5 detection means oriented to detect light from a region proximal to the waveguide, which includes:
a biosensor comprising:
a step gradient waveguide including a substrate formed of a first optical material of refractive index n_1 and having a surface disposed adjacent
10 and in contact with a waveguide film formed of a second optical material having a refractive index n_2 which is greater than n_1 ; and
at least one specific binding molecule immobilized to said waveguide film and constructed to bind with specificity an analyte.
- 15 2. The kit of Claim 1, wherein said second optical material is selected from the group consisting of: silicon oxynitride of refractive index n_2 , and deposited silicon dioxide; and said first optical material is selected from the group consisting of: deposited silicon dioxide, quartz or fused silica, silicon oxynitride of refractive index n_1 , and magnesium fluoride.
- 20 3. The kit of Claim 2, wherein said waveguide film is configured as a plurality of parallel longitudinal strips of said second optical material spaced from one another along said surface and constituting a plurality of waveguide channels, and further having a plurality of different species of said specific binding molecules
25 immobilized to said waveguide film, with each of said waveguide channels having a different one of said species immobilized thereto.
4. The kit of Claim 2, wherein said substrate surface has a grating formed thereon, said grating being constructed to facilitate coupling of light from an
30 incident excitation beam into said waveguide film.
5. The kit of Claim 1, wherein said waveguide film is coated with a coating which provides a level of nonspecific protein binding which is less than about 10%

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of the amount of specific binding of said analyte, said coating being selected from the group consisting of: polymethacryloyl polymer, polyethyleneglycol, and avidin; and wherein said capture molecules are immobilized to said waveguide film by binding to said coating.

5

6. The kit of Claim 1, wherein said waveguide is constructed by the steps of:

providing a piece of the first optical material having a surface;

vapor depositing the second optical material on said surface to a depth of between

10 about 0.1 μm and about 10 μm to produce said waveguide film;

coating at least one region of said waveguide layer with a resist compound which

resists an etchant to produce a coated region and an uncoated region; and

etching said waveguide layer with said etchant to remove said second optical material from said uncoated region.

15

7. The kit of Claim 6, wherein said second optical material is selected from the group consisting of: silicon oxynitride of refractive index n_2 , and silicon dioxide; and said first optical material is selected from the group consisting of: deposited silicon dioxide, quartz or fused silica, silicon oxynitride of refractive

20 index n_1 , and magnesium fluoride.

8. The kit of Claim 6, in whose manufacture said step of coating at least one region with a resist comprises coating a plurality of parallel longitudinal strips along said surface with said resist thereby producing a plurality of waveguide

25 channels, and wherein a plurality of different species of said specific binding molecules are immobilized to said waveguide film, with each of said waveguide channels having a different one of said species immobilized thereto.

9. The kit of Claim 8, whose construction further includes a step of etching

30 a plurality of spaced grooves into said surface of said substrate prior to said step of depositing said waveguide layer, wherein said grooves are oriented substantially perpendicular to said waveguide channels, and spacing of said grooves is selected to

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serve as a grating facilitating efficient coupling of light from an incident excitation beam into said sensor strips.

10. The kit of Claim 1, wherein said second optical material is silicon
5 oxynitride having an elemental ratio of approximately $\text{Si}_2\text{O}_3\text{N}$.

11. The kit of Claim 1, wherein said step gradient waveguide further includes a waveguide coupler contactingly disposed on said waveguide film, said waveguide coupler comprising:

10 an input waveguide formed of an optical material having a refractive index n_3 , and having a thickness which is between about 0.5 mm and about 5 mm; and a spacing layer formed of an optical material having a refractive index n_4 , wherein $n_4 < n_3$ and $n_4 < n_2$, and having a thickness selected to optimize the evanescent coupling of light from said input waveguide into said waveguide
15 film.

12. Apparatus for performing evanescent immunofluorescence assays, including:

a composite waveguide comprising:

20 a substrate formed of a first optical material of refractive index n_1 and having two parallel planar surfaces separated by a thickness and a surrounding edge, said thickness being between about 1 μm and about 10 mm, and

a waveguide film formed of a second optical material having a refractive
25 index n_2 which is greater than n_1 and a thickness which is between about 0.1 μm and about 10 μm ;

a light source operably disposed to aim a beam into said composite waveguide for propagation by total internal reflection therein;

light coupling means operably associated with said composite waveguide for

30 facilitating coupling of said light beam thereinto;

light collection means positioned to collect light that impinges on a plane spaced from said planar surfaces; and

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detection means operably disposed with respect to said light collection means for detecting said collected light.

13. The apparatus of Claim 12, wherein said first and second optical
5 materials are mutually selected from the group of pairwise combinations consisting of: deposited silicon dioxide/silicon oxynitride; quartz or fused silica/silicon oxynitride; silicon oxynitride of refractive index n_1 /silicon oxynitride of refractive index n_2 ; and magnesium fluoride/deposited silicon dioxide.

10 14. The apparatus of Claim 12, wherein said light coupling means is a waveguide coupler contactingly disposed on said waveguide film, and comprising: an input waveguide formed of an optical material having a refractive index n_3 , and having a thickness which is between about 0.5 mm and about 5 mm; and a spacing layer formed of an optical material having a refractive index n_4 , wherein
15 $n_4 < n_2$ and $n_4 < n_3$, and having a thickness selected to optimize the evanescent coupling of light from said input waveguide into said waveguide film.

15 15. The apparatus of Claim 13, wherein said waveguide film is constructed to have propagation losses in air of less than about 1 dB/cm.

25 16. An optical structure for performing specific binding assays with an optical unit comprising a light source positioned to direct light into the waveguide for propagation by total internal reflectance therein and detection means oriented to detect light from a region proximal to the optical structure, said optical structure comprising:

a substrate comprising a first optical material of refractive index n_1 and having two parallel planar surfaces separated by a thickness and a surrounding edge, said thickness being between about 0.3 μm and about 10 mm;
30 a waveguide film of a second optical material of refractive index n_2 in operable contact with at least a portion of one of said surfaces, said waveguide film having a thickness which is between about 0.3 μm and about 5 μm ; and

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coupling means integrally contacting said waveguide film for coupling light thereinto.

17. The optical structure of Claim 16, wherein said second optical material
5 is selected from the group consisting of: silicon oxynitride of refractive index n_2 and silicon dioxide; and said first optical material is selected from the group consisting of: pure silicon dioxide, quartz or fused silica, silicon oxynitride of refractive index n_1 , and magnesium fluoride.

10 18. The optical structure of Claim 17, wherein said coupling means is selected from the group consisting of: an optical grating formed of said first material; an optical grating formed of said second material; and a waveguide coupler integrally mounted on said waveguide film, said waveguide coupler comprising
15 an input waveguide constructed to receive light through an edge and to propagate the received light by total internal reflection, and a spacing layer interposed between said input waveguide and said waveguide film and having a thickness selected to optimize the evanescent coupling of light from said input waveguide into said waveguide film.

20 19. The optical structure of Claim 16, wherein said first optical material is quartz, fused silica or deposited SiO_2 , said coupling means is said waveguide coupler, and wherein said input waveguide is formed of an optical material selected from the group consisting of: rutile, zirconia, and high-index glass; and wherein
25 said spacing layer is formed of silicon dioxide and has a thickness of between about $0.1\ \mu\text{m}$ and about $5\ \mu\text{m}$.

20. The optical structure of Claim 16, wherein said waveguide film is configured as plurality of strips of said second optical material adhered to one of
30 said surfaces in a spaced parallel array.

21. The optical structure of Claim 16, which further includes a plurality of sample wells formed thereon, said sample wells formed by the steps of:

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epitaxially depositing an outer layer of refractive index n_1 upon said waveguide film;
applying a resist compound to a plurality of outlines which define said sample wells while leaving areas within and without the outlines uncoated; and
5 removing said outer layer by etching to expose said waveguide film in said uncoated areas.

22. A method of making a waveguide biosensor for performing specific binding assays with an optical unit including a light source positioned to direct light
10 into the optical structure for propagation by total internal reflectance therein and detection means oriented to detect light from a region proximal to the optical structure, said optical structure constructed by the steps of:
providing a substrate comprising a first optical material of refractive index n_1 and having two parallel planar surfaces separated by a thickness and a
15 surrounding edge;
depositing a waveguide layer of a second optical material having a refractive index n_2 on one of said surfaces, said waveguide layer having a thickness which is between about $0.1\ \mu\text{m}$ and about $10\ \mu\text{m}$ and n_2 being greater than n_1 ;
coating at least one region of said waveguide layer with a resist compound which
20 resists an etchant to produce a coated region and an uncoated region;
etching said waveguide layer with said etchant to remove said second material from the uncoated region;
removing the resist compound from the etched waveguide to expose the coated region; and
25 immobilizing a plurality of capture molecules to said exposed coated region, said capture molecules being constructed to bind with specificity a selected analyte molecule.

23. The method of Claim 22, further including a step of coating said
30 exposed coated region with a coating which provides a level of nonspecific protein binding which is less than about 10% of the amount of specific binding of said analyte, said coating being selected from the group consisting of: polymethacryloyl

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polymer, polyethyleneglycol, and avidin; and wherein in said step of immobilizing capture molecules, said capture molecules are bound to said coating.

24. The method of Claim 23, wherein said second optical material is
5 selected from the group consisting of: silicon oxynitride of refractive index n_2 , and deposited silicon dioxide; and said first optical material is selected from the group consisting of: deposited silicon dioxide, quartz or fused silica, silicon oxynitride of refractive index n_1 , and magnesium fluoride.

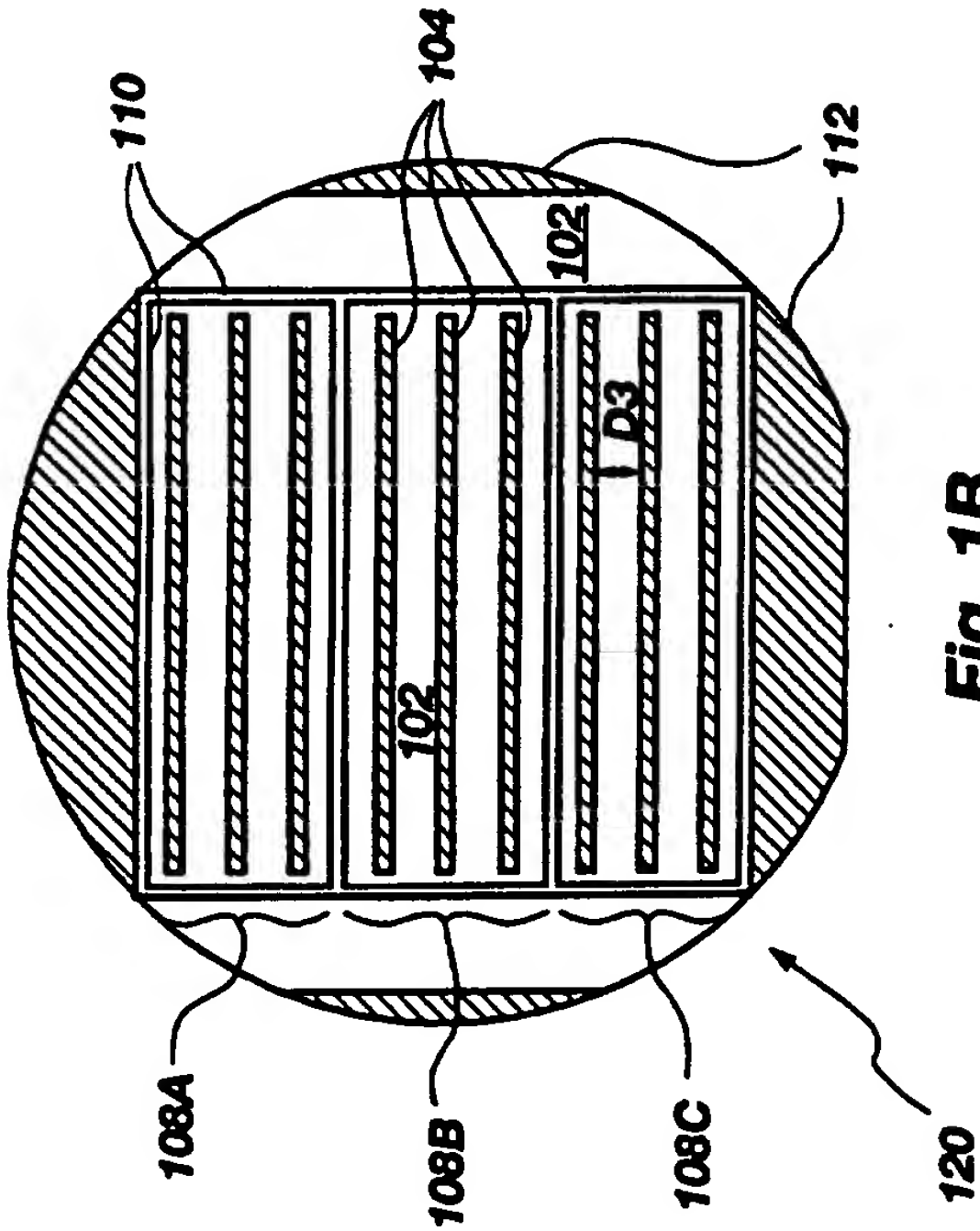


Fig. 1B

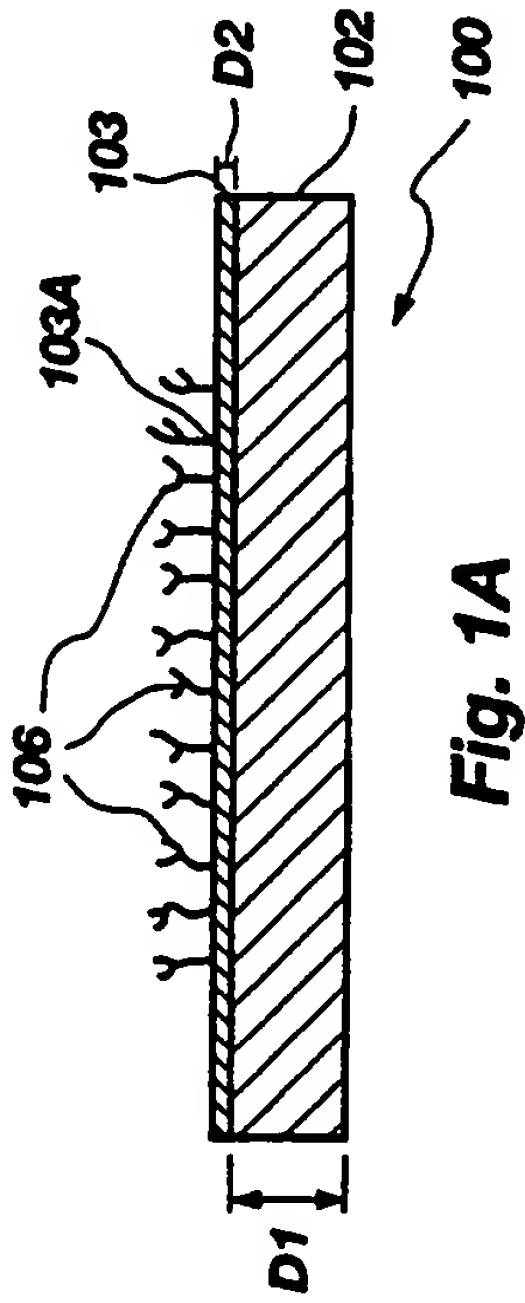


Fig. 1A

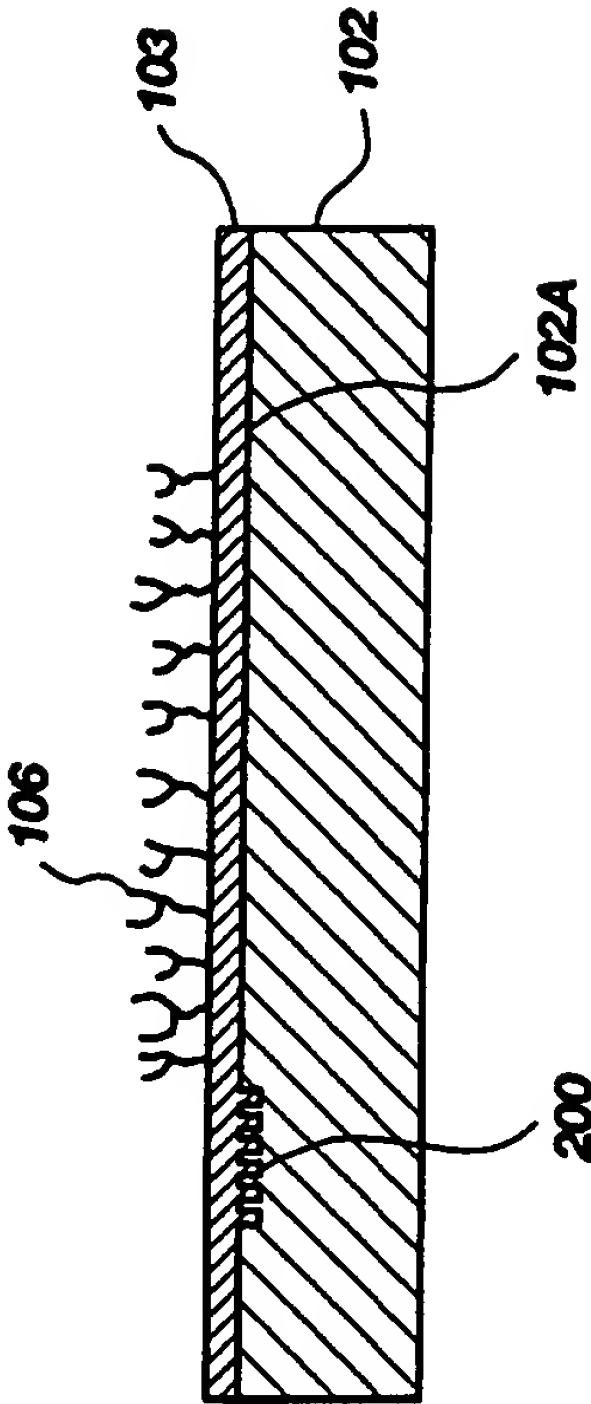


Fig. 2A

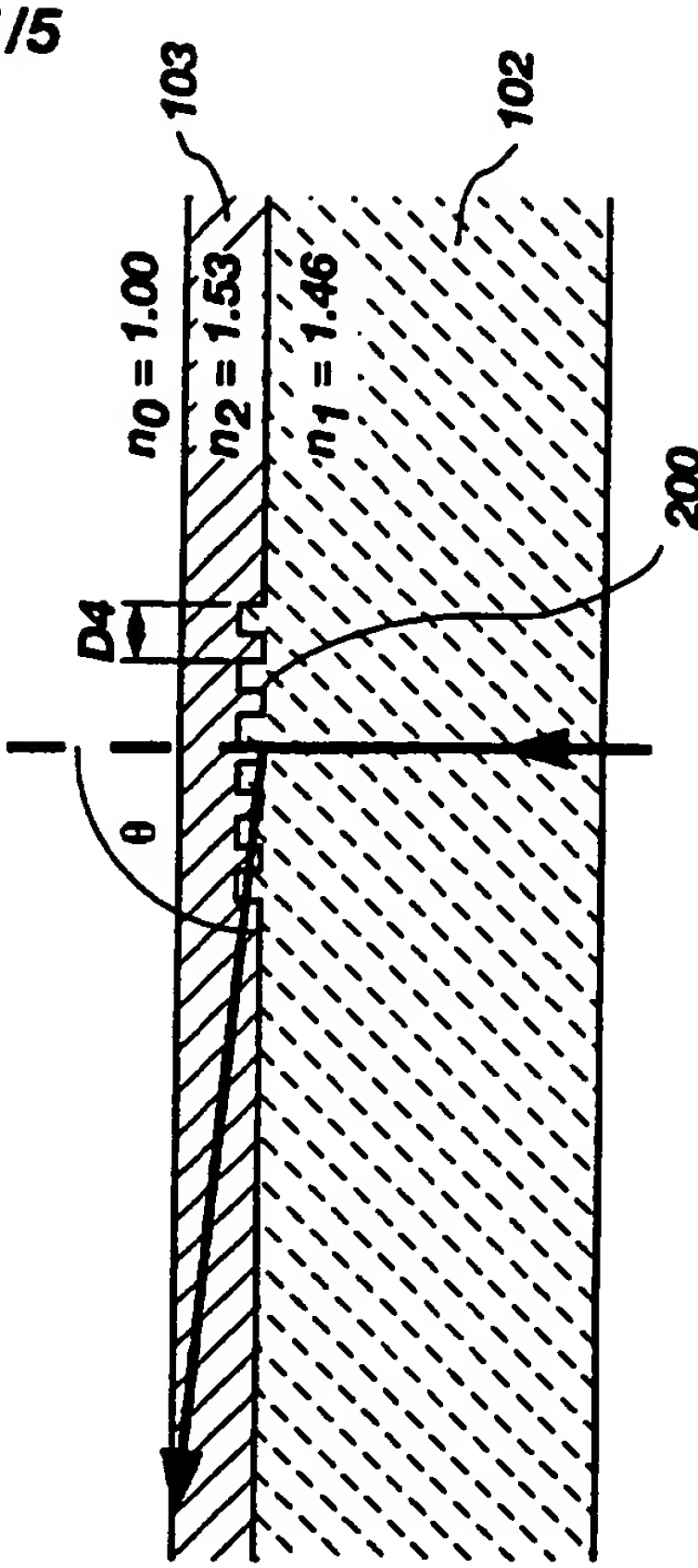


Fig. 2B

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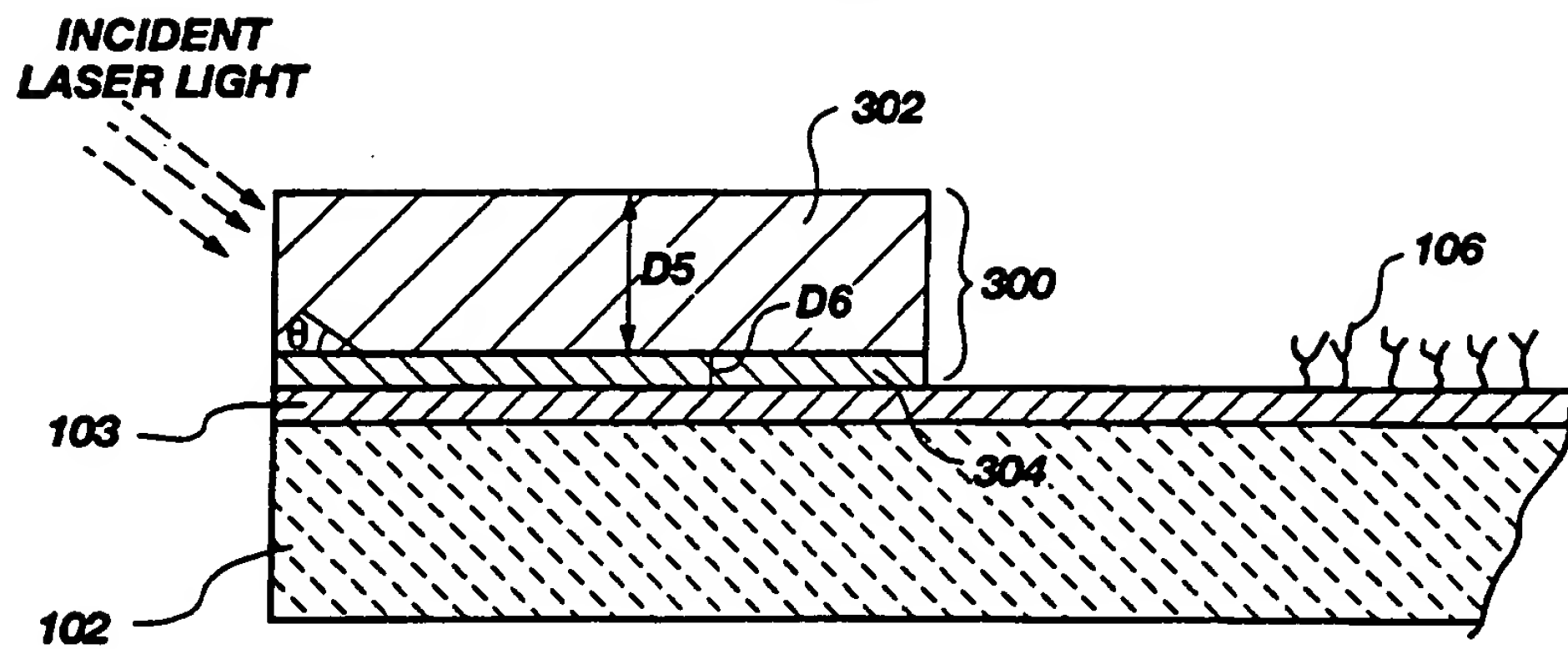


Fig. 3A

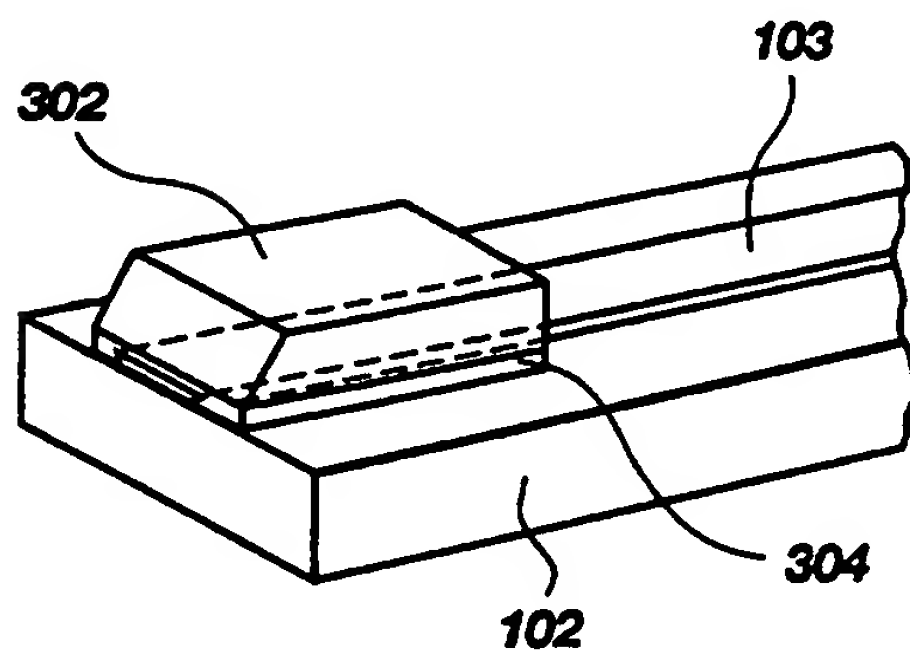


Fig. 3B

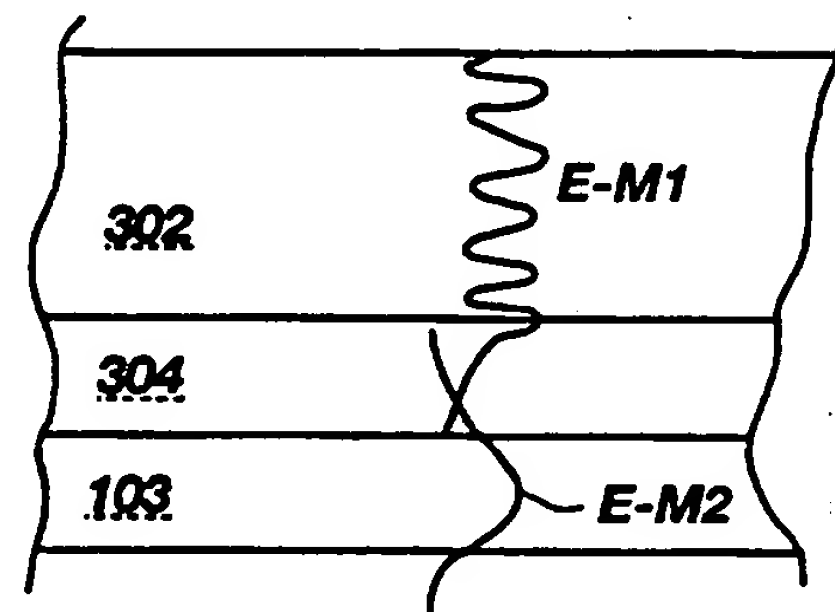
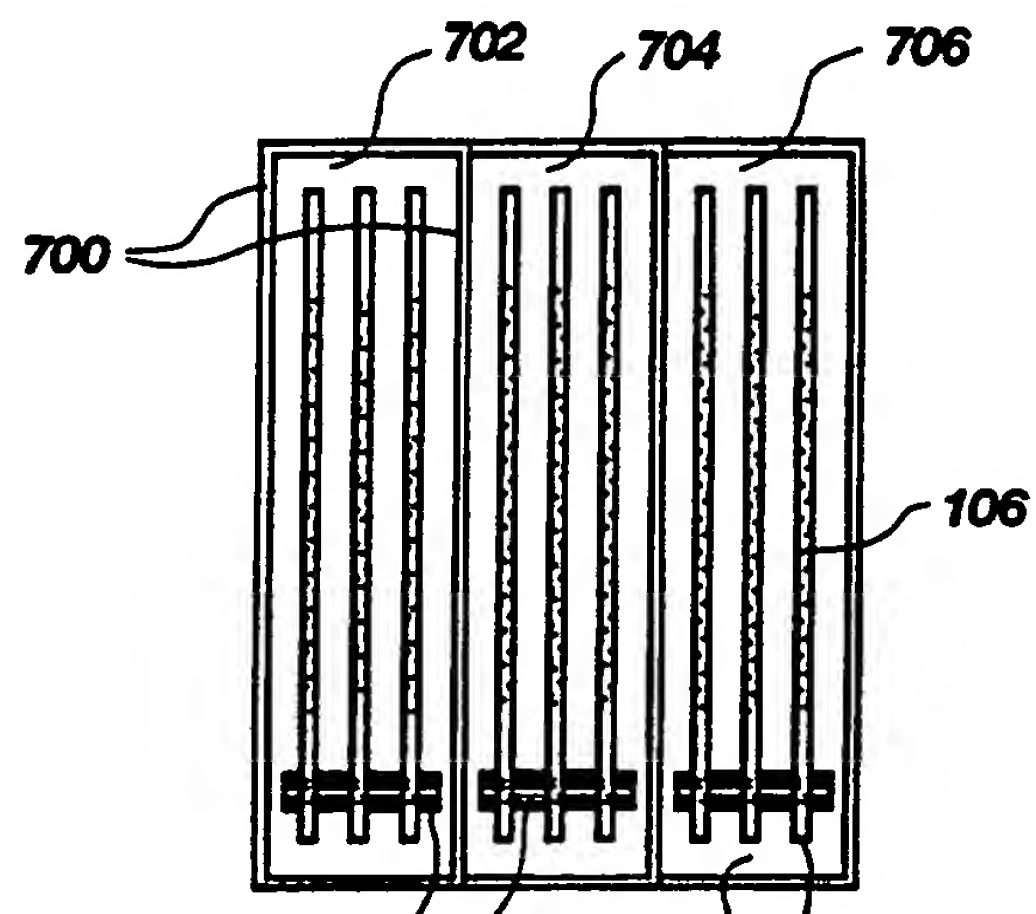


Fig. 3C



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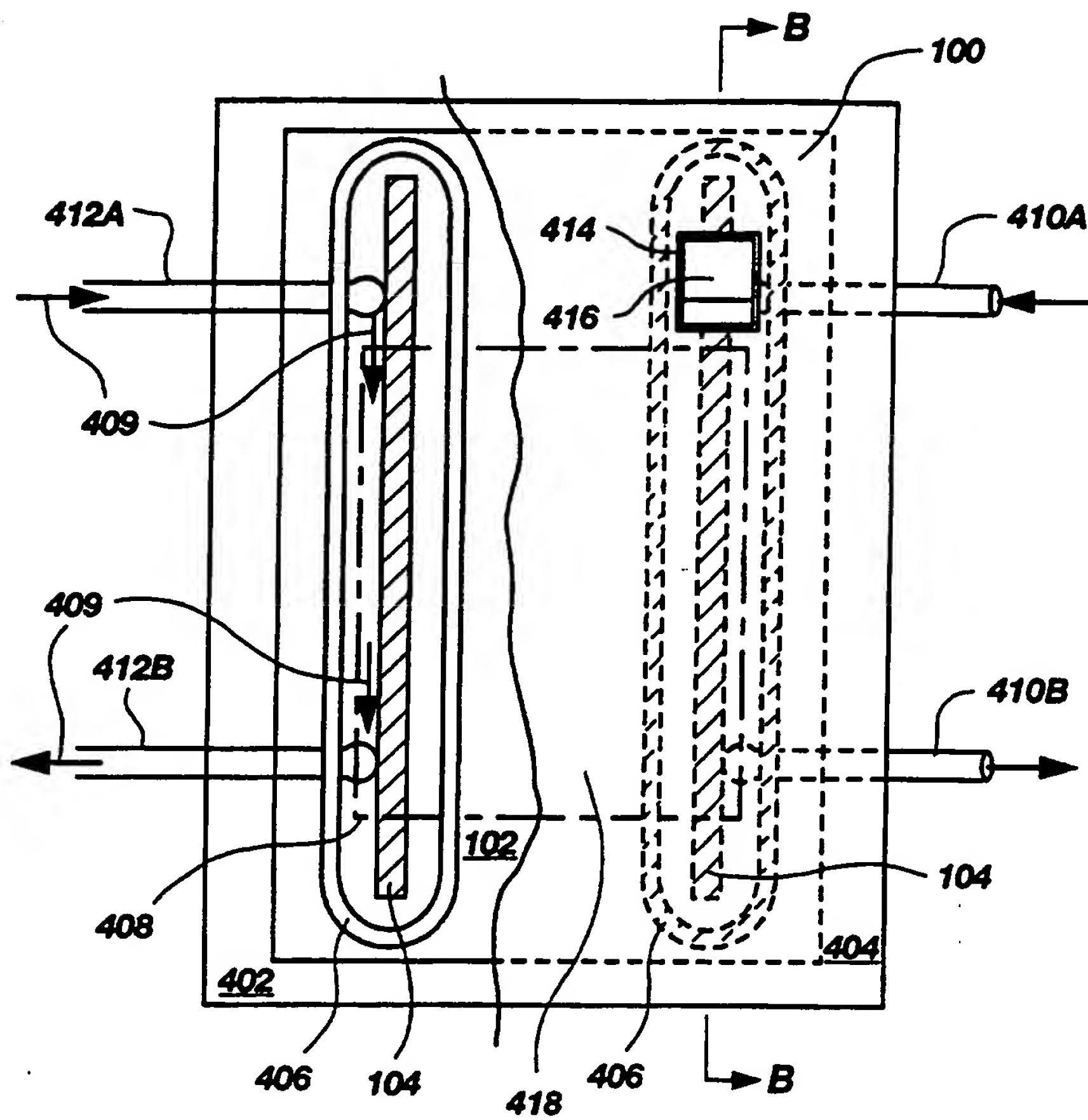


Fig. 4A

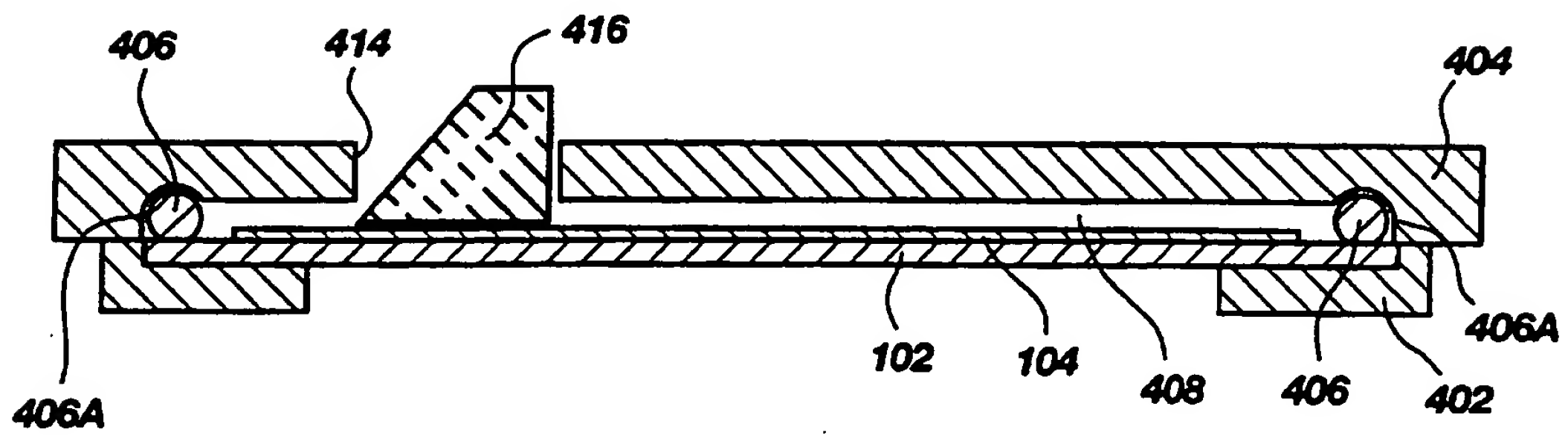
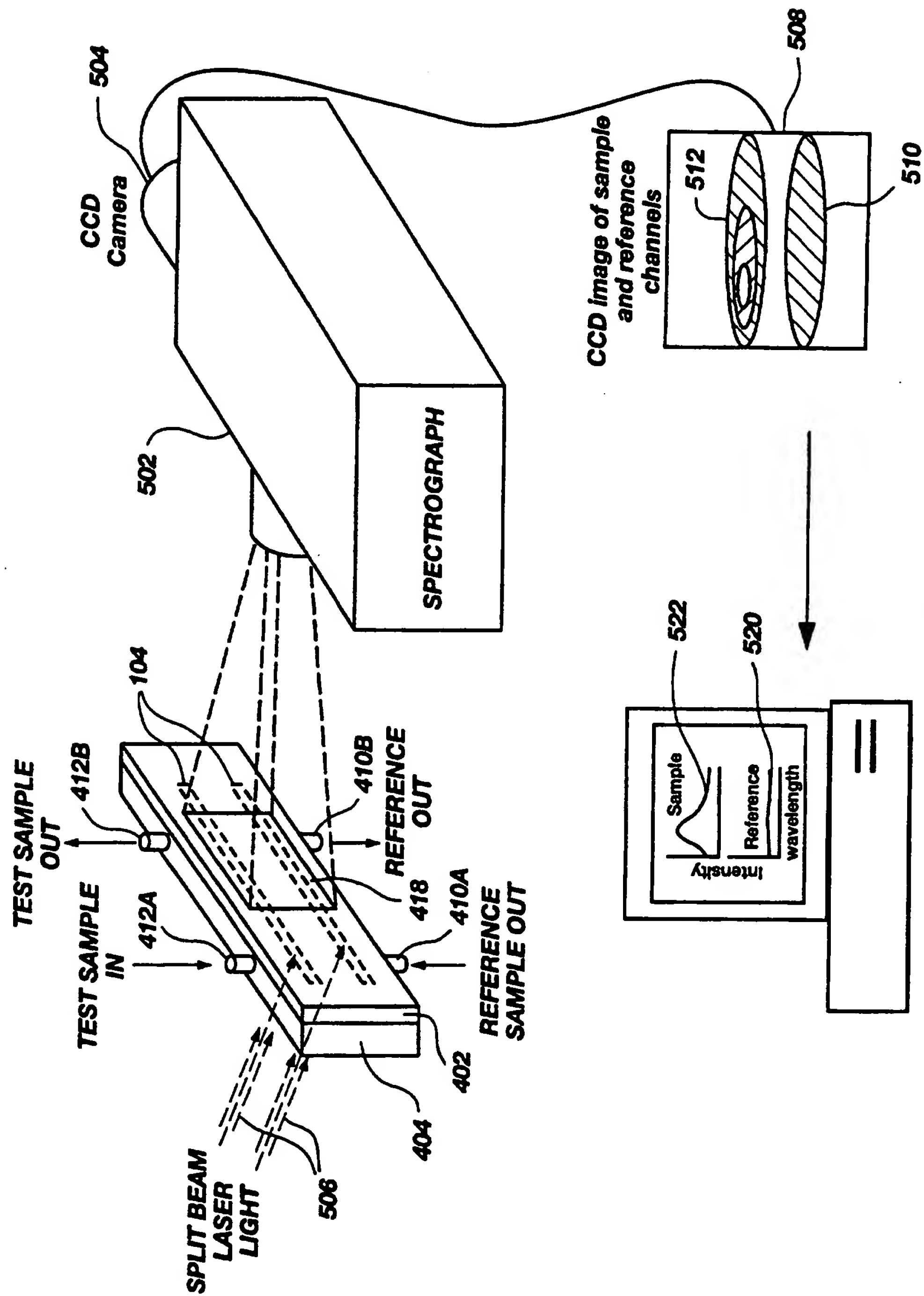


Fig. 4B



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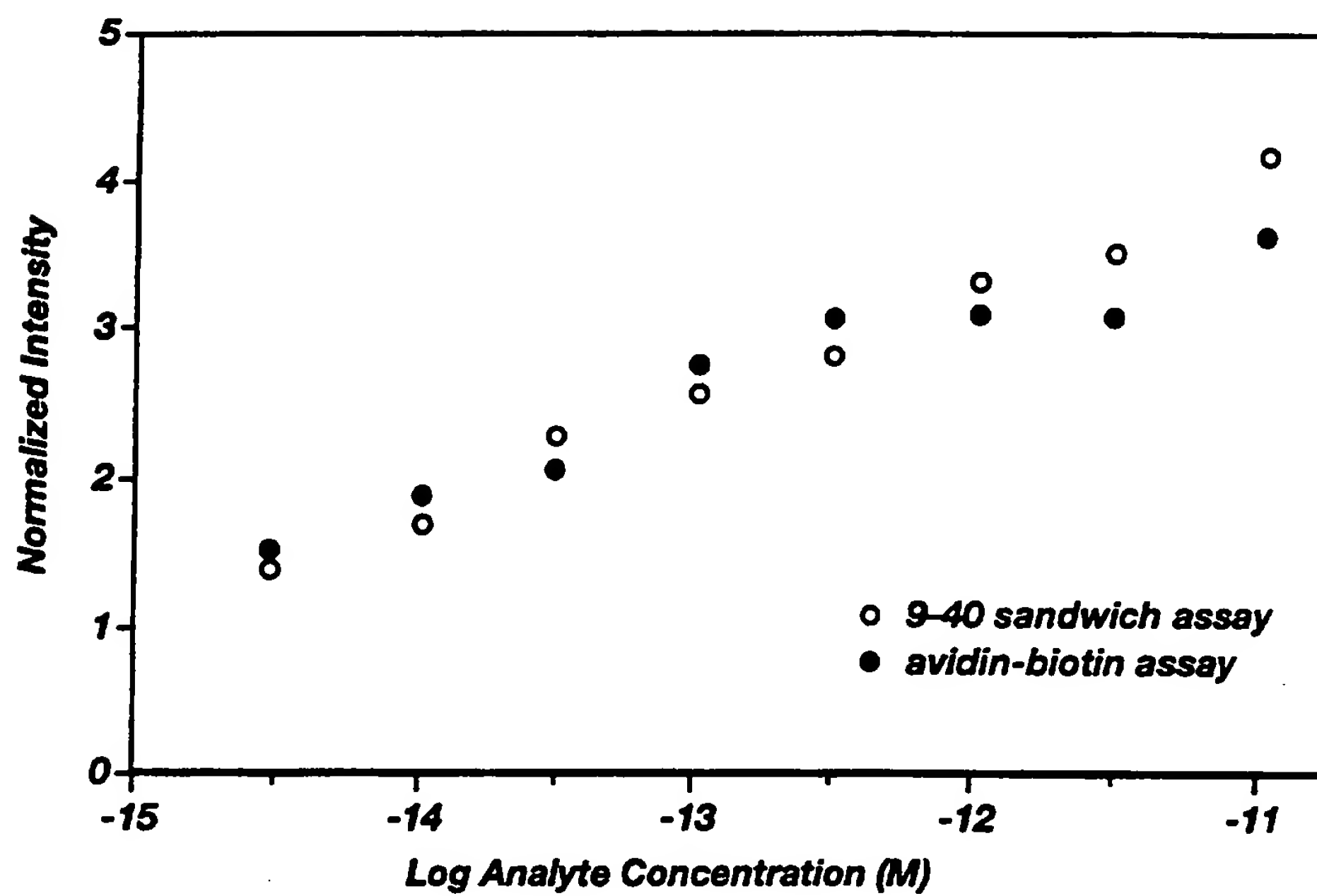


Fig. 6A

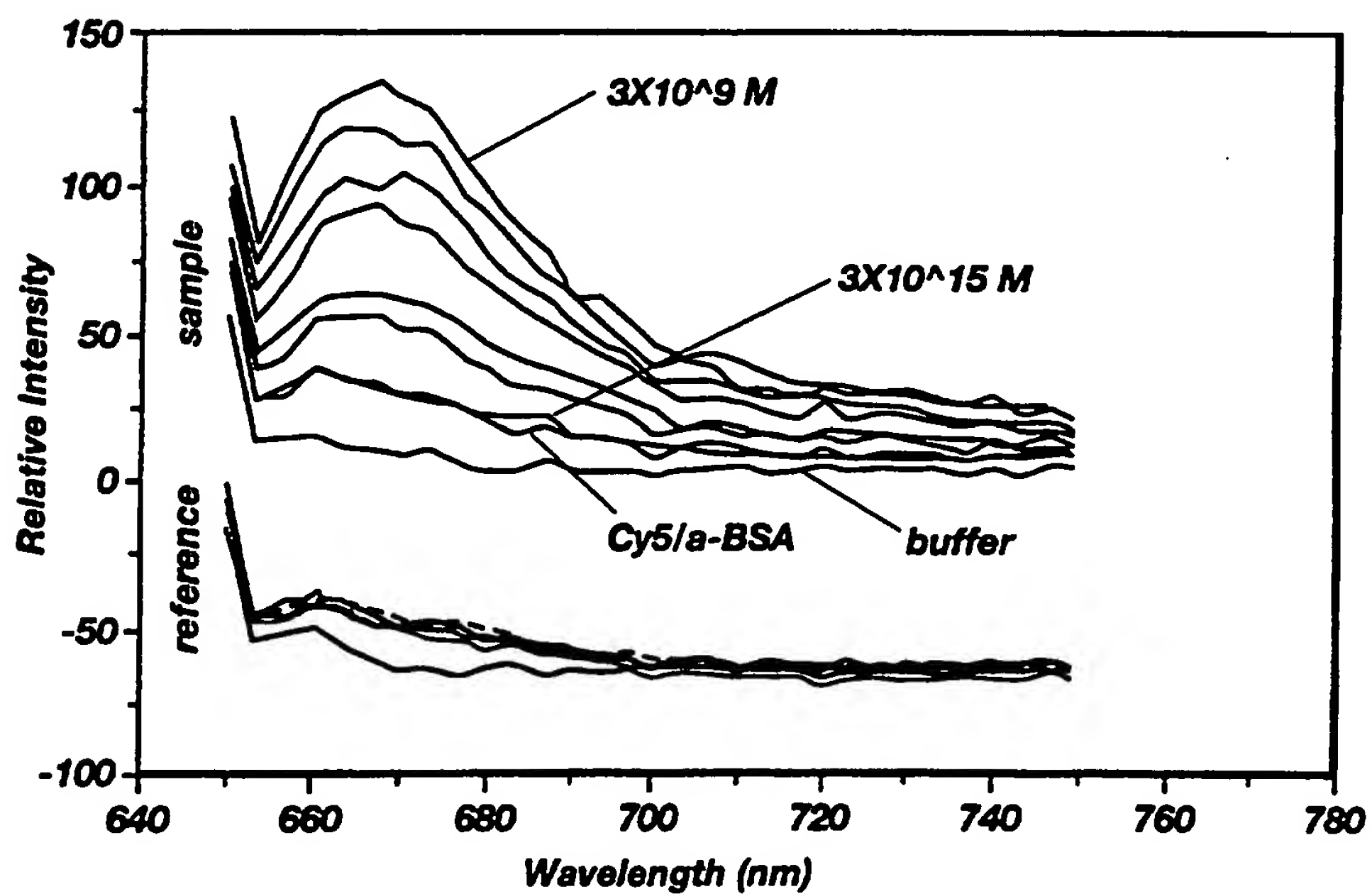


Fig. 6B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/02662

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : G01N 21/64; G01N 33/532

US CL : 422/82.05; 436/525

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 422/82.05, 82.06, 82.07, 82.08, 82.09, 82.11; 436/525; 385/12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
noneElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)
none

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US,A, 4,880,752 (KECK ET AL.) 14 NOVEMBER 1989, see col. 6 lines 13 +	1,12,16 ----- 2-11, 13-15 and 17-24
X -- Y	US,A, 5,344,784 (ATTRIDGE) 6 SEPTEMBER 1994, columns 2-4	1,12,16 ----- 2-11, 13-15 and 17-24



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	* T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A* document defining the general state of the art which is not considered to be of particular relevance	* X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* E* earlier document published on or after the international filing date	* Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* Z	document member of the same patent family
* O* document referring to an oral disclosure, use, exhibition or other means		
* P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

27 JUNE 1996

Date of mailing of the international search report

23 JUL 1996

Name and mailing address of the ISA/US
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Box PCT
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Facsimile No. (703) 305-7719

Authorized officer

LYLE A. ALEXANDER *get*

Telephone No. (703) 308-3893